

# ***Saccharomyces cerevisiae* protein phosphatase 2A performs an essential cellular function and is encoded by two genes**

**Alan A.Sneddon, Patricia T.W.Cohen and Michael J.R.Stark**

Department of Biochemistry, The University, Dundee DD1 4HN, UK  
Communicated by P.Cohen

Two genes (*PPH21* and *PPH22*) encoding the yeast homologues of protein serine-threonine phosphatase 2A have been cloned from a *Saccharomyces cerevisiae* genomic library using a rabbit protein phosphatase 2A cDNA as a hybridization probe. The *PPH* genes are genetically linked on chromosome IV and are predicted to encode polypeptides each with 74% amino acid sequence identity to rabbit type 2A protein phosphatase, indicating once again the extraordinarily high degree of sequence conservation shown by protein phosphatases from different species. The two *PPH* genes show <10% amino acid sequence divergence from each other and while disruption of either *PPH* gene alone is without any major effect, the double disruption is lethal. This indicates that protein phosphatase 2A activity is an essential cellular function in yeast. Measurement of type 2A protein phosphatase activity in yeast strains lacking one or other of the genes indicates that they account for most, if not all, protein phosphatase 2A activity in the cell.

**Key words:** cell cycle/protein phosphatase/*Saccharomyces cerevisiae*/yeast

## **Introduction**

The phosphorylation state of any protein depends on the opposing actions of protein kinases and protein phosphatases. Although the regulation of protein function by reversible phosphorylation is a well-known phenomenon, until recently research has focused more on the roles of specific protein kinases in cellular control mechanisms. This has perhaps been due to the perception of protein phosphatases as a small group of rather broad specificity enzymes. However, recent work in genetically tractable systems, such as yeast, has started to indicate that protein phosphatases, like protein kinases, play important and specific roles in cellular regulation (reviewed in Cyert and Thorner, 1989).

The catalytic subunits of protein serine-threonine phosphatases have been classified into four types (1, 2A, 2B and 2C) on the basis of substrate specificity, sensitivity to inhibitors and divalent cation requirements (Cohen, 1989; Cohen and Cohen, 1989). Comparison of amino acid sequence data for all four types from mammalian sources has indicated that the first three are related, although type 1 (PP1) and type 2A (PP2A) show the greatest sequence similarity

(Cohen and Cohen, 1989). Recently, we have demonstrated activities with closely similar biochemical properties to these four types in extracts of *Saccharomyces cerevisiae* (Cohen *et al.*, 1989b).

The isolation of two *S.cerevisiae* and two *Schizosaccharomyces pombe* protein phosphatase genes has been reported previously. *S.cerevisiae* *SIT4* (*PPH1*) encodes a polypeptide with sequence similarities to both mammalian PP1 (43% identity) and PP2A (60% identity). *sit4* mutations permit the expression of *HIS4*, a gene required for histidine biosynthesis, in the absence of the normally required transcriptional activators encoded by *GCN4*, *BAS1* and *BAS2* (Arndt *et al.*, 1989). *GCN4* mediates activation of *HIS4* (and many other genes) in response to amino acid starvation, while *BAS1* and *BAS2* regulate basal level control of *HIS4* expression in response to phosphate and adenine levels in the growth medium (Arndt *et al.*, 1987). *sit4* mutations also affect the expression of a number of other genes and show genetic interactions with *sit1* and *sit2* mutant alleles of the *RPB1* and *RPB2* genes encoding subunits of RNA polymerase II (Arndt *et al.*, 1989). The *SIT4* product has therefore been inferred to play a role in the regulation of transcriptional initiation. In contrast, *dis2*<sup>+</sup>, an *S.pombe* gene which plays a role in the segregation of sister chromatids during mitosis, has recently been shown to encode a type 1 protein phosphatase (PP1). Together with a related *S.pombe* sequence (*sds21*<sup>+</sup>), *dis2*<sup>+</sup> is one of a pair of PP1 genes which between them are essential for viability in fission yeast (Ohkura *et al.*, 1989). *S.cerevisiae* contains a homologue of the *dis2*<sup>+</sup> gene (*DIS2S1*; Ohkura *et al.*, 1989), although its role in budding yeast has yet to be described. Deletion of either *SIT4* (Cyert and Thorner, 1989) or *DIS2S1* (our unpublished data) is lethal, indicating that both these protein phosphatases encode essential functions in *S.cerevisiae*. A mutation in a gene (*bimG*) similar to *dis2*<sup>+</sup>/*DIS2S1* blocks completion of mitosis in *Aspergillus* (Doonan and Morris, 1989), while null mutations in one *Drosophila* PP1 gene also result in defective mitosis (Axton *et al.*, 1990; Dombrádi *et al.*, 1990). Comparison of these various PP1 amino acid sequences has revealed extreme sequence conservation throughout evolution, greater even than for histones 2A, 2B and cyclic AMP dependent protein kinase (Cohen, 1990), PP1 from sources as disparate as rabbit and yeast showing >82% identity.

PP1 therefore plays important roles in the control of the mitotic cell cycle. However, work using *Xenopus* eggs has indicated that PP2A is also involved in the control of mitotic activation (Félix *et al.*, 1990). We have therefore sought to identify *S.cerevisiae* gene(s) encoding PP2A in order to examine the role of PP2A activity in cell cycle regulation. Here we report the isolation and characterization of the two *S.cerevisiae* PP2A genes, *PPH21* and *PPH22*, which between them encode an activity essential for cell viability.

## Results

### Isolation of the *PPH21* and *PPH22* genes

In view of the apparently high sequence conservation between protein phosphatase genes from different sources, we first attempted to identify *S.cerevisiae* genomic sequences related to PP2A by hybridization of yeast genomic Southern blots with a probe derived from a rabbit PP2A cDNA (da Cruz e Silva *et al.*, 1987). This demonstrated the existence of at least one related sequence and indicated suitable conditions of stringency for obtaining a positive signal ( $5 \times \text{SSC}$  at  $48^\circ\text{C}$ ; data not shown). Screening of a yeast cDNA library constructed in  $\lambda\text{gt}10$  (U.Präkelt and P.Meacock, unpublished) using the same probe and conditions resulted in the isolation of a number of potential positive clones. In view of the sequence similarities shown by mammalian PP1 and PP2A sequences, we wished to have some criterion for distinguishing possible PP1 and PP2A clones. We therefore screened the positive  $\lambda\text{gt}10$  clones by Southern hybridization of their inserts at the same stringency using the *Aspergillus nidulans* *bimG* PP1 gene (Doonan and Morris, 1989) as a probe. Of five clones thus screened, only one failed to hybridize to this probe. DNA sequencing subsequently indicated that this latter clone (AS3c) encoded a novel yeast protein phosphatase while the other four sequences were identical with the genomic sequence of *DIS2S1* (Ohkura *et al.*, 1989), the *S.cerevisiae* homologue of the *dis2*<sup>+</sup> PP1 gene.

When the insert from AS3c was used to probe a yeast genomic Southern blot (see below), it became clear that the yeast genome contained a second AS3-related sequence, both hybridizing sequences being located on  $\sim 4.5$  kb *Xba*I restriction fragments. We therefore constructed a mini-library of *S.cerevisiae* *Xba*I restriction fragments of this size and screened it by hybridization using the AS3c insert as a probe. This yielded a genomic clone for both AS3 (AS3g) and the second sequence (termed AS6g). Figure 1 shows restriction maps of these two genomic inserts.

### Sequence analysis of *PPH1* and *PPH2*

Southern blot analysis of the AS3g and AS6g inserts using the AS3c insert as a probe indicated that, in each case, the hybridizing sequences were located towards the leftmost end of the insert as depicted in Figure 1. The complete nucleotide sequence of the appropriate region of each clone was

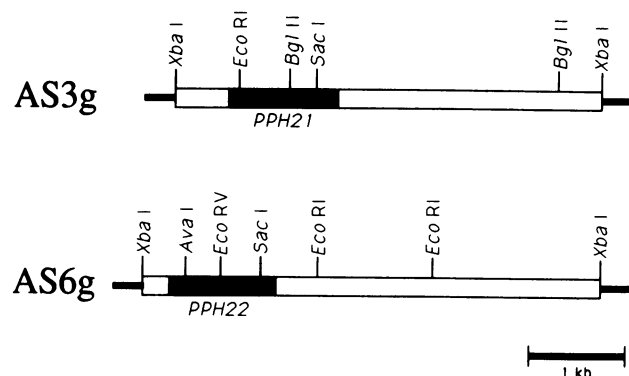


Fig. 1. Restriction maps of the yeast genomic DNA carried by clones AS3g and AS6g. Flanking vector sequence is represented by narrower black bars and the location of important restriction sites is shown. The positions of the *PPH21* and *PPH22* coding regions are denoted by shading.

therefore determined and is presented in Figure 2. In each case, inspection of the sequence revealed the presence of a long open reading frame (ORF) of 369 (AS3) or 377 (AS6) amino acids with extensive sequence similarity to the rabbit PP2A polypeptide (Figure 3, Table I). In view of these data and the biochemical evidence presented below, we have therefore termed the genes encoded by AS3g and AS6g *PPH21* and *PPH22* respectively (for protein phosphatase 2A 1 and 2). No evidence of the consensus sequences for the donor, TACTAAC or acceptor sites indicative of *S.cerevisiae* introns (see Gallwitz *et al.*, 1987) was found in either genomic sequence. Using *PPH* gene specific probes, we detected specific transcripts of  $\sim 1.5$ – $1.9$  kb corresponding to each *PPH* gene in poly(A)<sup>+</sup> RNA. The two transcripts differed somewhat in size, the *PPH22* mRNA being longer, but were expressed at approximately similar levels (data not shown).

The two *PPH* genes show  $>87\%$  sequence identity within the predicted coding regions. Indeed, the genes themselves share a region of 194 bp of uninterrupted sequence identity. However, the flanking DNA sequences show considerable divergence. The deduced *PPH* amino acid sequences are  $>90\%$  identical and within the carboxy-terminal 325 residues there are only five conservative and three non-conservative changes (Figure 3). Less than half the codons used are those favoured by highly expressed yeast genes (Bennetzen and Hall, 1982), consistent with relatively low level expression suggested by the very weak signal obtained on Northern blot analysis of total RNA (not shown). Table I indicates that both predicted *PPH* polypeptides show greatest identity to the rabbit PP2A sequence, with lower levels of relatedness to PP1 from rabbit and yeast sources and to rabbit PPX and PPV. PPX (da Cruz e Silva *et al.*, 1988) and PPV (Cohen *et al.*, 1990) encode protein phosphatases more closely related to PP2A than to PP1 which were isolated by cloning but whose biochemical properties are as yet unknown. The similarity of *PPH21* and *PPH22* to rabbit PP2A is much greater than that shown by *SIT4*, a potential yeast PP2A gene identified previously (Arndt *et al.*, 1989). The data in Figure 3 show that the PP2A similarity is entirely contained within the carboxy-terminal 300 residues of the predicted *PPH* polypeptides: thus both *PPH21* and *PPH22* appear to encode amino-terminal extensions of 60–68 residues relative to their mammalian counterpart. Indeed, the predicted *PPH* products are considerably longer than any other known PP1 or PP2A polypeptide. In contrast, PP1 from yeasts and mammals are of very similar predicted size (Ohkura *et al.*, 1989). Figure 3 shows that while part of these extensions is highly conserved between the two yeast genes, the extreme amino-terminal region is not.

Inspection of the two *PPH* ORFs indicates the presence of three (*PPH21*) or four (*PPH22*) potential translation initiation codons upstream of the region conserved between the yeast and mammalian PP2A sequences. However, we believe that the correct initiation site is likely to be the first of these potential ATG codons. In neither case is the sequence ATG found further upstream in the sequences which we have determined. Although the DNA sequences are relatively divergent in this 5' region of the two *PPH* genes, they are nevertheless extremely conserved around the first ATG (Figure 4), providing the best AUG context of the possible start codons (see Hamilton *et al.*, 1987). In addition, our isolation (in the case of *PPH21*) of a cDNA

extending to within nine bases of the first ATG is fully consistent with the notion that the complete predicted amino-terminal extensions are real, while any alternative interpretation would require the unfavourable presence of at least one upstream AUG codon in the mRNA.

### *S.cerevisiae* contains no other closely related protein phosphatase genes

Southern blot analysis of the yeast genome at moderate to high stringency using either *PPH* gene as a probe indicated

the presence of only two strongly hybridizing sequences in a variety of restriction digests (Figure 5 and data not shown). By using very high stringency ( $0.1 \times \text{SSC}$  at  $70^\circ\text{C}$ ) or probes specific for one or other *PPH* gene (see Materials and methods) it was possible to determine which bands corresponded to which *PPH* gene. Even at lower stringency ( $5 \times \text{SSC}$  at  $55^\circ\text{C}$ ), no other sequences which hybridized to the *PPH* probes were observed (not shown). We therefore conclude that there are no other genes in *S.cerevisiae* which are closely related to *PPH21* and *PPH22*.

## B

## A

**HpaII**

1 CCGGTTGTGTTTTT GAAATAAGGCCAAAG AGAAAGAAAAAACA AATTATTTTGGCCCTT  
61 ATTTAGTTTCATAGTG GAAAGAGGGATATAA ATTATCGCATAAAC AATAAACAAAAAGAA

1 Met Asp Thr Asp Leu Asp Val Pro Met Gln Asp Ala Val Thr Glu  
121 AA ATG GAT ACA **↑** GAT TTA GAT GTG CCT ATG CAA GAT GCT GTT ACC GAA

16 Gln Leu Thr Pro Thr Val Ser Glu Asp Met Asp Leu Asn Asn Asn Ser  
168 CAG CTG ACT CCC ACA GTA TCT GAA GAT ATG GAT CTC AAC AAC AAT TCA

32 Ser Asp Asn Asn Ala Glu Phe Ser Val Asp Asp Leu Lys Pro Gly  
216 TCG GAT AAT AAT GCA GAA **GAA TTC** TCG GTT GAT GAT TTA AAA CCT GGT

48 Ser Ser Gly Ile Ala Asp His Lys Ser Ser Lys Pro Leu Glu Leu Asn  
264 TCC TCG GGC ATA GCA GAT CAC AAA TCT TCC AAA CCA CTA GAA CTG AAT

64 Asn Thr Asn Ile Asn Gln Leu Asp Gln Trp Ile Glu His Leu Ser Lys  
312 AAC ACA AAT ATA AAT CAG CTT GAC CAA TGG ATT GAG CAT TTG AGT AAA

80 Cys Glu Pro Leu Ser Glu Asp Asp Val Ala Cys Arg Leu Cys Lys Met Ala  
360 TGC GAG CCA CTA TCA GAA GAT GAT GAA CTA TGT AAA ATT GCG

96 Val Asp Val Leu Gln Phe Glu Glu Asn Val Lys Pro Ile Asn Val Pro  
408 GTG GAC GTG TTG CAG TTC GAG GAG AAT GTT AAA CCA ATT AAC GTG CCT

112 Val Thr Ile Cys Gly Asp Val His Gln Phe His Asp Leu Leu Glu  
456 GTT ACC ATT TGT GGT GAC TTA CAC GGT CAA TTC CAT GAC TTG TTA GAA

128 Leu Phe Lys Ile Gly Gly Pro Cys Pro Asp Thr Asn Tyr Leu Phe Met  
504 CTT TTT AAG ATT GGT GGC CCT TGT CCT GAC ACC AAT TAC CTT TTC ATG

144 Gly Asp Tyr Val Asp Arg Gly Tyr Tyr Ser Val Glu Thr Val Ser Tyr  
552 GGT GAT TAC GTG GAT AGA GGA TAT TAT TCT GTT GAA ACT GTA TCT TAC

160 Leu Val Ala Met Lys Val Arg Tyr Pro His Arg Ile Thr Ile Leu Arg  
600 CTA GTT GCC ATG AAA GTC AGA TAC CCA CAT AGA ATT ACG ATA CTG AGA

176 Gly Asn His Glu Ser Arg Gln Ile Thr Gln Val Tyr Gly Phe Tyr Asp  
648 GGC AAT CAC GAG TCT AGG CAG ATT ACC CAA GTA TAC GGG TTT TAT GAT

192 Glu Cys Leu Arg Lys Tyr Gly Ser Ala Asn Val Trp Lys Met Phe Thr  
696 GAA TGT TTG AGA AAG TAC GGC AGT GCA AAG GTG TGG AAA ATT TTC ACA

208 Asp Leu Phe Asp Tyr Phe Pro Ile Thr Ala Leu Val Asp Asn Lys Ile  
744 **GAT CTT** TTC GAT TAT TTT CCT ATA ACC GCA TTA GTA GAT AAT AAA ATT

224 Phe Cys Leu His Gly Gly Leu Ser Pro Met Ile Glu Thr Ile Asp Gln  
792 TTC TGT CTG CAT GCA GGA CTT TCC CCC ATG ATA GAA ACC ATA GAT CAG

240 Val Arg Glu Leu Asn Arg Ile Gln Glu Val Pro His Glu Gly Pro Met  
840 GTG AGA GAG TTG AAC AGA ATA CAG GAA GTG CCT CAT GAA GGT CCT ATG

256 Cys Asp Leu Leu Trp Ser Asp Pro Asp Asp Arg Gly Gly Trp Gly Ile  
888 TGT GAC CTT CTA TGG TCA GAC CCT GAC GAT AGA GGC GGA TGG GGA ATC

272 Ser Pro Arg Gly Ala Gly Phe Thr Phe Gly Gln Asp Val Ser Glu Gln  
936 AGT CCC AGA GGT GCA GGC TTC ACT TTT GGA CAA GAT GTC AGT GAG CAA

288 Phe Asn His Thr Asn Asp Leu Ser Leu Ile Ala Arg Ala His Gln Leu  
984 TTC AAT CAC ACT AAT GAT CTA TCA CTA ATA GCA **AGA GCT** CAC CAA TTG

304 Val Met Glu Gly Tyr Ala Trp Ser His Gln Gln Asn Val Val Thr Ile  
1032 GTA ATG GAA GGC TAT GCT TGG TCT CAC CAG CAA AAT GTT GTC ACC ATT

320 Phe Ser Ala Pro Asn Tyr Cys Gly Asn Gln Ala Ala Ile  
1080 TTC AGT GCT CCT AAT TAC TGC TAC AGA TGT GGT AAT CAA GCA GCT ATC

336 Met Glu Val Asp Glu Asn His Asn Arg Gln Phe Leu Gln Tyr Asp Pro  
1128 ATG GAA GTG GAG AAT CAT AAT AGA CCA TTC TTA CAG TAC GAG CCA

352 Ser Val Arg Pro Gly Glu Pro Ser Val Ser Arg Lys Thr Pro Asp Tyr  
1176 TCC GTA AGA CCA GGT GAA CCT AGC GTC AGC AGA AAG ACG CCA GAT TAC

368 Phe Leu \*\*\*  
1224 TTT TTA TGA GTA TGTATACATATATG ATCTATATAGATATA TATTCACCTTTCTCC

1281 TTTACC

1 TCTAGAGAGGGTTAT TATTTCTTTTCCGC TGAATTGTGGAAAAG AGTCGTGGAAAGTTT  
XbaI  
61 CAAAGGGACATCCCC ATTTTATAAAACCAA TTGAAGGGCTGTAAG TAGAGCAAACTCTT  
121 AAGGTACACGCCCGC TGC AAAACAGTGTTC GAACAAGAACACGCA AACCCGTTTACTTCT  
KpnI  
181 TGTCTCACTGTCTCT CTTTCTCTTCGCGTT ATTGTTCTCTGTACG TCTTTTTTGAATTT

1 Met Asp Met Glu  
241 TATATTATTGGCACT TCTGTATAACTGGCT TTCATTCGAAAAA ATG GAT ATG GAA

5 Ile Asp Asp Pro Met His Gly Ser Asp Glu Asp Gln Leu Ser Pro Thr  
297 ATT GAT GAC CCT ATG CAT GGT TCA GAT GAA GAT CAA TTA TCA CCG ACT

21 Leu Asp Glu Asp Met Asn Ser Asp Asp Gly Lys Asn Asn Thr Lys Ala  
345 CTC GAC GAA GAC ATG AAT AGT GAT GAC GGC AAA AAT AAT ACG AAG GCG

37 Arg Ser Asn Asp Glu Asp Thr Asp Glu Glu Leu Glu Asp Phe Asn Phe  
393 CGT TCT AAT GAC GAA GAC ACA GAT GAA GAG TTG GAA GAT TTT AAT TTT

53 Lys Pro Gly Ser Ser Gly Ile Ala Asp His Lys Ser Ser Lys Pro Leu  
441 AAA CCG GGG TCC **TGC GGT** ATA GCA GAT CAT AAA TCC TCC AAA CCA CTA  
DraI  
489 Lys Leu Thr Asn Thr Asn Ile Asn Gln Leu Asp Gln Trp Ile Glu His  
489 AAA CTG ACC AAT ACA AAT ATA AAT CAG CTT GAC CAA TGG ATT GAG CAT

85 Leu Ser Lys Cys Glu Pro Leu Ser Glu Asp Asp Val Ala Arg Leu Cys  
537 TTG AGT AAA TGC GAG CCA CTA TCA GAA GAC GAT GTA GCA CGA CTA TGT

101 Lys Met Ala Val Asp Val Leu Gln Phe Glu Glu Asn Val Lys Pro Ile  
585 AAA ATG GCG GTG GAC GTG TTG CAG TTC GAG GAG AAT GTT AAA CCA ATT

117 Asn Val Pro Val Thr Ile Cys Gly Asp Val His Gly Gln Phe His Asp  
633 AAC GTG CCT GTT ACC ATT TGT GGT GAC GTA CAC GGT CAA TTC CAT GAC

133 Leu Leu Glu Leu Phe Lys Ile Gly Gly Pro Cys Pro Asp Thr Asn Tyr  
681 TTG TTA GAA CTT TTC AAG ATT GGT GGT CTT TGT CCT GAC ACC AAT TAC

149 Leu Phe Met Gly Asp Tyr Val Asp Arg Gly Tyr Tyr Ser Val Glu Thr  
729 CTT TTC ATG GGT GAT **TAC GTG** GAT AGA GGA TAT TAT TCT GTT GAG ACC  
BsaAI  
165 Val Ser Tyr Leu Val Ala Met Lys Val Arg Tyr Pro His Arg Ile Thr  
777 GTA TCT TAC CTA GTT GCC ATG AAA GTC **AGA TAT** CCA CAT AGA ATT ACT

181 Ile Leu Arg Gly Asn His Glu Ser Arg Gln Ile Thr Gln Val Tyr Gly  
825 ATA CTT AGG GGC AAT CAC GAG TCT AGG CAG ATT ACC CAA GTA TAT GGG

197 Phe Tyr Asp Glu Cys Leu Arg Lys Tyr Gly Ser Ala Asn Val Trp Lys  
873 TTT TAT GAC GAA TGT TTG AGA AAG TAC GGC AGT GCG AAC GTG TGG AAA

213 Met Phe Thr Asp Leu Phe Asp Tyr Phe Pro Val Thr Ala Leu Val Asp  
921 ATG TTT ACC GAT CTA TTC GAT TAT TTC CCC GTT ACT GGC TTG GTG GAT

229 Asn Lys Ile Phe Cys Leu His Gly Gly Leu Ser Pro Met Ile Glu Thr  
969 AAT AAA ATC TTC TGT TTG CAT GGA GGT CTC TCA CCC ATG ATA GAG ACA

245 Ile Asp Gln Val Arg Asp Leu Asn Arg Ile Gln Glu Val Pro His Glu  
1017 ATA GAT CAA GTT AGA **GAT TTA AAT** AGA ATA CAG GAA GTG CCT CAC GAA

261 Gly Pro Met Cys Asp Leu Leu Trp Ser Asp Pro Asp Asp Arg Gly Gly  
1065 GGT CCA ATG TGT GAC CTT CTA TGG TCC GAT CCT GAT GAT AGA GGC GGA

277 Trp Gly Ile Ser Pro Arg Gly Ala Gly Phe Thr Phe Gly Gln Asp Ile  
1113 TGG GAA ATC AGT CCG AGA GGT GCA GGC TCT ACT PTT GGT CAA GAC ATC

293 Ser Glu Gln Phe Asn His Thr Asn Asp Leu Ser Leu Ile Ala Arg Ala  
1161 AGT GAG CAA TTC AAT CAC ACT AAT GAC CTA TCA CTA ATA GCA **AGA GCT**  
SacI  
309 His Gln Leu Val Met Glu Gly Tyr Ser Trp Ser His Gln Gln Asn Val  
1209 CAC CAA TTG GTA ATG GAA GGA TAT TCT TGG TCT CAC CAG CAA AAT GTT

325 Val Thr Ile Phe Ser Ala Pro Asn Tyr Cys Tyr Arg Cys Gly Asn Gln  
1257 GTC ACC ATT TTC AGT GCT CCA AAT TAT TGT TAT AGA TGT GGT AAC CAG

341 Ala Ala Ile Met Glu Val Asp Gln Asn His Asn Arg Gln Phe Leu Gln  
1305 GCC GCT ATT ATG GAG GTG GAT GAA AAC CAT AAT AGG CAA TTC TTA CAA

357 Tyr Asp Pro Ser Val Arg Pro Gly Glu Pro Thr Val Thr Arg Lys Thr  
1353 TAC GAT CCA TCT GTG AGA CCC GGT GAA CCA ACC GTC ACC AAG ACA

373 Pro Asp Tyr Phe Leu \*\*\*  
1401 CCG GAT TAT TTC TTA TAA TATATAT CTATTACACCTTTAT CCTTACTACGCTATT

1456 TCATTCCACATACA TTTATTTTTTTTTT TTTTAAATATATAT TAGTTACTTTTCCC  
1516 TCTAATTTTTTGATAA TAACAACCTATATAC AAACATACAAACTAT TTTTCGTTTTTCAAA  
1576 TACTATTTTTTCGA ACACAAAGTGAACAC AACTTTTTCTCATAT ATTACTGTTCTCATTA  
1636 CTAGATTTCTATATA ACCGTTACGTAATTT TTTTCGTTATGATAG TTCACTCCTCAGCGT

1696 AAATGTTTAATTTAT CAAATTTCACTCTCA CCGAATCAGTATCCC TAGATGACGGCGAAC  
1756 CCTCAACACTAGGCC **TACGAATT C**  
EcoRI

Fig. 2. *PPH21* and *PPH22* nucleotide sequences. The complete nucleotide sequences of *PPH21* (A) and *PPH22* (B) are presented together with flanking regions. The predicted *PPH* gene products are presented above the DNA sequences and the positions of important restriction sites shown. In (A) the small arrow denotes the first nucleotide of the AS3 cDNA, which also included a short poly(A) tail attached to C<sub>1286</sub>.

### Null *PPH* alleles are viable but the double *PPH* disruption is lethal

In order to test whether the *PPH* genes encode essential cellular functions, we next constructed diploid strains containing *pph* null alleles and performed tetrad analysis. Figure 6 shows the *pph21::LEU2* and *pph22::URA3* constructions used in these experiments. First, a linear DNA fragment carrying *pph21::LEU2* was introduced into the yeast genome by transformation of MSY111 to leucine prototrophy, yielding ASY100 (Table V). On sporulation and subsequent tetrad analysis, all nine tetrads analysed gave four viable spores and showed 2:2 segregation of both *leu*<sup>+</sup> and of all other markers tested. Thus the ability to obtain

PPH21	1	MDTDLDPVMDAVTEQLTPTVSEDM...DLNNSDDNNAEEFVDDL...	44
PPH22	1	MDMEIDDPHMGSDQLSPTLDEDMNSDDGKNNTKARSDNEDTDEELED	50
PPH21	45	..KPGSSGIADHKSSKPLENNNTINQLDQWIEHLSKCEPLSEDDVARLC	92
PPH22	51	NFKPGSSGIADHKSSKPKLTNTINQLDQWIEHLSKCEPLSEDDVARLC	100
PP2A	1	.....MDEKVFTELQDQWIEHLSKCEPLSEDDVARLC	32
pph21	93	KMAVDVLQFEENVKPINVPVTICGDVHGQFHDLELFKIGGCPDNTNLYF	142
PPH22	101	KMAVDVLQFEENVKPINVPVTICGDVHGQFHDLELFKIGGCPDNTNLYF	150
PP2A	33	EKAKEILTKESNVQEVRCPTVCVGDVHGQFHDLELFKIGGCPDNTNLYF	82
PPH21	143	MGDYVDRGYYSVETVSYLVAMKVRYPHRITILRGNHESRQITQVGYFYDE	192
PPH22	151	MGDYVDRGYYSVETVSYLVAMKVRYPHRITILRGNHESRQITQVGYFYDE	200
PP2A	83	MGDYVDRGYYSVETVSYLVAMKVRYPHRITILRGNHESRQITQVGYFYDE	132
PPH1	193	CLRKYSANVWKMFTDLDFYFPITALVDNKFICLHGLSPMIETIDQVRD	242
PPH22	201	CLRKYSANVWKMFTDLDFYFPITALVDNKFICLHGLSPMIETIDQVRD	250
PP2A	133	CLRKYSANVWKMFTDLDFYFPITALVDNKFICLHGLSPMIETIDQVRD	182
PPH21	243	LNRIQEVPHGPMCDLLWSPPDRGGWGISPRGAGFTFGQDVSEQFNHTN	292
PPH22	251	LNRIQEVPHGPMCDLLWSPPDRGGWGISPRGAGFTFGQDVSEQFNHTN	300
PP2A	183	LDRLQEVPHGPMCDLLWSPPDRGGWGISPRGAGFTFGQDVSEQFNHTN	232
PPH21	293	DLSLIARAHQLVMEGYAWSHQQNVVTFISAPNYCYRCGNQAAIMEVDENH	342
PPH22	301	DLSLIARAHQLVMEGYAWSHQQNVVTFISAPNYCYRCGNQAAIMEVDENH	350
PP2A	233	GLTLVSRAHQLVMEGYAWSHQQNVVTFISAPNYCYRCGNQAAIMEVDENH	282
PPH21	343	NRQFLQYDPSVRPGEPVSARKTPDYFL	369
PPH22	351	NRQFLQYDPSVRPGEPVTTRKTPDYFL	377
PP2A	283	KYSFLQFDPAPRRGEPVTRRTPDYFL	309

**Fig. 3.** Optimal alignment of the predicted *PPH* gene products and rabbit PP2A. The *PPH* polypeptides and the rabbit PP2A amino acid sequence (da Cruz e Silva *et al.*, 1987) were aligned using the UWGCG program GAP (Devereux *et al.*, 1984). Identical residues (|) and conservative changes (○) are indicated.

**Table I.** Relatedness of protein phosphatase amino acid sequences

	Rabbit PP2A	Rabbit PP1	Rabbit PPX	Drosophila PPV	S.cerevisiae DIS2S1	S.cerevisiae SIT4
PPH21	74	43	58	51	42	54
PPH22	74	43	58	50	42	54
SIT4	58	43	60	61	42	100
DIS2S1	43	81	45	42	100	42

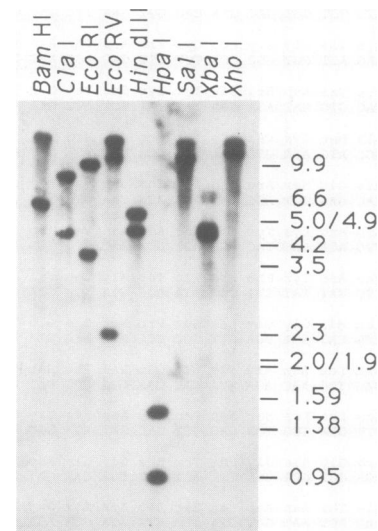
Pairwise comparisons of sequences were made using the UWGCG program GAP (with the GAP and LENGTH parameters set to 2.5 and 0.1 respectively) in order to derive a percentage similarity of the sequences. Except for PP1 (Berndt *et al.*, 1987), the source of each sequence is cited in the text.

viable *leu*<sup>+</sup> spores clearly indicated that *PPH21* is not an essential gene.

However, given the extraordinary degree of similarity between the two *PPH* genes, it was considered highly likely that they would have overlapping or even identical functions within the cell. Disruption of either gene alone might therefore be tolerable by the cell even if the two genes

1	.....CCGGTTGTGTTTTTGAATAAGGCAAAAGAGAAAGAAAAACAAATTA	71
150	CGAACAAAGAACACGCAAAACCGTTACTTCTTGCTACCTGCTCTCTTCTTCCGGT	209
51	TTTTGCCCTTATTAGTTCATAGTGGAA...AGAGGGATATAAAATATCGATAAAACAA	107
210	TATTGTCTCTGTACGCTCTTTTTTGAATTTATATTATGGCCTCTGTATACTGGC	269
108	TAAACAAAAAGAAATGATACAGATTAGATGTGCTTATCAAGATGCTGTACCGAA	167
270	TTTCATTCGAAAAATGATATGGAATTTATGACCTATCATGGTTCAGATGAAGAT	329
	MetAspThrAspLeuAspValProMetGlnAspAlaValThrGlu	
168	CAGCTGACTCCACAGATATCTGAAGATATG.....GATCTCAACAAATTCATCG	218
330	CAATTATCACCAGCTCTCGACGAAGATGATAGTAGTACGCGCAAAATATACGAAG	389
	GlnLeuThrProThrValSerGluAspMet.....AspLeuAsnAsnSerSer	
219	GATAATATGCAGAGATTTCTCGGTTGATGATTAA.....AAACCTGGT	263
390	GCGCGTCTAATGACGAAGACAGATGAAGAGTTGGAAGATTTAATTTAAACCGGGG	449
	AlaArgSerAsnAspGluAspThrAspGluLeuGluSerAspGlyLysAsnThrLys	
264	TCCTCGGGCAGATACAGATACAAATCTTCCAAACCTAGAACTGAATAACAAATATA	323
450	TCCTCGGGTATAGCAGATCAAAATCTTCCAAACCTAAACCTGACCAATACAAATATA	509
	SerSerGlyIleAlaAspHisLysSerSerLysProLeuGluLeuAsnAsnThrAsnIle	

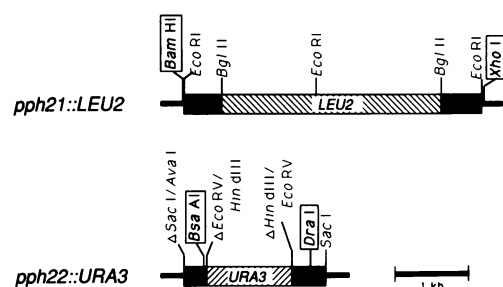
**Fig. 4.** Conservation of DNA sequence around the start of the *PPH* genes. The DNA sequences encompassing the amino-terminal regions of *PPH21* and *PPH22* were aligned using the UWGCG program GAP (Devereux *et al.*, 1984). The two predicted amino acid sequences are shown and the positions encoding in-frame AUG codons boxed. Numbering of the sequences is as in Figure 2.



**Fig. 5.** Southern hybridization of yeast genomic DNA restriction digests with a *PPH* probe. Digests of yeast genomic DNA prepared with the restriction enzymes indicated were separated by agarose gel electrophoresis, transferred to a nylon membrane and probed using a radiolabeled *EcoRI* fragment of the AS3c cDNA clone of *PPH21*. This extended from the *EcoRI* site within *PPH21* to an *EcoRI* site in the Bluescript vector located nine bases to the right of the sequence presented in Figure 2A. Hybridization was performed in 5 × SSC at 65°C, under which conditions the probe hybridizes strongly to fragments carrying either *PPH* gene. The positions to which size standards migrated are indicated (sizes in kb). In every digest except *EcoRV* (which cuts within the *PPH21* hybridizing region; see Figure 1), just two hybridizing bands were apparent.

encoded an essential activity. We therefore performed a second experiment in which the *pph22::URA3* disruption was introduced into the genome of ASY100 by transformation to uracil prototrophy with a suitable fragment of DNA (see Figure 6). This generated a diploid strain (ASY101) now heterozygous at both the *PPH21* and *PPH22* loci (Table V). On sporulation and tetrad analysis, out of 56 tetrads analysed, over half had at least one dead spore and  $leu^+ ura^+$  spores were never obtained. From the segregation pattern of the *leu* and *ura* markers, every dead spore was deduced to have been  $leu^+ ura^+$  (Table II). This clearly indicates that the double *pph21 pph22* null is lethal. However,  $leu^- ura^+$  spores indicative of a *PPH21 pph22::URA3* genotype were readily obtainable, indicating that deletion of just the *PPH22* gene alone was, like the *pph21* null allele, tolerable by the cell. We therefore conclude that the two *PPH* genes which we have isolated encode between them a function which is essential to the yeast cell.

The data in Table II also indicate genetic linkage of the two *PPH* genes. For unlinked genes, the two ditype classes should be represented in approximately equal numbers (see Mortimer and Schild, 1982). Since our data indicate a vast excess of one of the two ditype classes, both *PPH* genes must therefore be located on the same chromosome. This was confirmed by using *PPH21* and *PPH22* gene specific probes to examine a Southern blot of *S.cerevisiae* chromosomes separated by CHEF electrophoresis, which indicated that both sequences were located on chromosome IV (using the *SEC1* gene as a control probe). From our data we calculate the *PPH21*–*PPH22* linkage distance to be ~35 centimorgans and conclude that the *pph* disruptions carried by ASY101 are located on different chromosome IV homologues.



**Fig. 6.** Production of null *pph21*<sup>o</sup> and *pph22*<sup>o</sup> alleles. Restriction maps depicting the *pph21::LEU2* and *pph22::URA3* constructs used to make strains deleted for one or other *PPH* gene are shown. Flanking vector sequences are indicated by narrower black bars and the restriction sites used to generate linear DNA fragments for yeast transformation are boxed.

By inspection of the spores which failed to grow, we hoped to determine whether they had germinated and then arrested at a particular stage of the cell division cycle. However, examination of the 'dead' spores indicated a variety of phenotypes ranging from the presence of single, large unbudded cells to microcolonies of ~32 cells, including both budded and unbudded cells. It was therefore impossible to ascertain in this way whether the double *pph* null cell was blocked at a specific stage in the yeast cell cycle.

#### Strains carrying null *PPH* alleles have reduced PP2A activity

Haploid strains carrying either the *pph21::LEU2* or the *pph22::URA3* single null alleles generated from the experiment summarized in Table II were examined for PP2A activity *in vitro*. PP2A activity in extracts of strains derived from tetratype tetrads [each providing one wild-type (w.t.), one *pph21* and one *pph22* spore] were compared, using mammalian glycogen phosphorylase as a substrate. PP2A activity was defined as that which was divalent cation independent and inhibitable by okadaic acid at 2 nM, as described previously (Cohen *et al.*, 1989a,b). Data from experiments using two such series of strains indicated that the specific activity of PP2A was reproducibly lower when one of the two *PPH* genes was disrupted, while there was no significant change in the specific activity of PP1 (Table III). Taken together with the strong sequence similarity to rabbit PP2A, there can therefore be little doubt that both *PPH21* and *PPH22* are structural genes for PP2A catalytic subunits. Table III shows that while *PPH21* accounts for 51% of the total PP2A activity, *PPH22* accounts for just 33%. The relative contribution of *PPH21* to the total is therefore greater by a factor of ~1.5.

#### Null *PPH* alleles are without detectable phenotype

Haploid *pph21::LEU2 PPH22* and *PPH21 pph22::URA3* strains were next examined for any phenotype(s) indicative of specific roles for PP2A in the yeast cell. Given that the single null strains had significantly reduced PP2A activity (above), it was anticipated that some differences might be detectable between such strains and strains w.t. at both *PPH* loci. As Table IV shows, no major differences between w.t., *pph21::LEU2* or *pph22::URA3* strains could be found when growth rate, percent budding, sensitivity to the mating pheromone  $\alpha$ -factor or heat-shock sensitivity were examined. However, the *pph21::LEU2* and *pph22::URA3* strains grew slightly more slowly and had a significantly lower budding index than the control *PPH*<sup>+</sup> strains, suggesting that the *pph* strains have a slightly lengthened G<sub>1</sub> phase. While all

**Table II.** Meiotic segregation pattern of *pph21::LEU2* and *pph22::URA3* on sporulation of ASY101

Ascus type	'Parental' ditype	'Non-parental' ditype	Tetratype
Properties of spores	4 viable spores (2 $leu^+ ura^-$ and 2 $leu^- ura^+$ )	2 viable spores (both $leu^- ura^-$ )	3 viable spores (1 $leu^- ura^-$ , 1 $leu^+ ura^-$ and 1 $leu^- ura^+$ )
Number of asci	22	1	33

The extreme bias in the relative numbers of ditype asci indicates linkage of the two markers (see text) and allowed assignment of the first class as 'parental' ditype asci (i.e. not requiring meiotic recombination to be generated). This enabled calculation of a linkage distance of 35 centimorgans using the formula

$$cM = 100 \left[ \frac{T + 6 \times NPD}{2 \times \text{total asci}} \right]$$

(see Mortimer and Schild, 1982).

three types of strain could grow on non-fermentable carbon sources, strains carrying either *pph* null allele grew less vigorously than the *PPH21 PPH22* controls. Since failure to grow on non-fermentable carbon sources is associated with activation of the adenylate cyclase pathway in yeast (see e.g. Toda *et al.*, 1987a), this might point towards a role for yeast PP2A in opposing some of the effects of cAMP dependent protein kinase. In summary, cells lacking one of the two PP2A genes show little change in phenotype with respect to growth, cell division or response to mating pheromone. However, since the two *PPH* genes are clearly essential for cell viability, we conclude that they must be largely or even completely equivalent functionally and that PP2A activity is not particularly limiting to the cell for any of the properties examined.

## Discussion

### Are there further PP2A genes in *S.cerevisiae*?

We have isolated and characterized two novel PP2A genes from *S.cerevisiae* and our data indicate that *PPH21* and

**Table III.** Specific activity of PP2A and PP1 in extracts of yeast strains

Genotype	PP2A activity (mU/mg)	PP1 activity (mU/mg)	Percentage loss PP2A activity
<i>PPH21 PPH22</i>	0.252 ± 0.041	0.181 ± 0.032	—
<i>pph21::LEU2 PPH22</i>	0.124 ± 0.016	0.165 ± 0.026	51
<i>PPH21 pph22::URA3</i>	0.168 ± 0.031	0.162 ± 0.026	33

Assays were performed as described (Materials and methods). All three progeny from two tetratype tetrads (each providing one w.t., one *pph21* and one *pph22* spore) were examined and the mean values (expressed as milliunits/mg protein) obtained for the two strains of each *PPH* genotype in two separate assays. The percentage loss of PP2A activity is also indicated. A milliunit is defined as 1 nmol substrate dephosphorylated/min/ml enzyme.

**Table IV.** Properties of strains lacking *PPH21* or *PPH22* function

Genotype	$\alpha$ -factor sensitivity zone size (mm)			Doubling time (min)	Budding index (%)	Heat-shock sensitivity	Growth on non-fermentable carbon sources
	5 $\mu$ g	10 $\mu$ g	20 $\mu$ g				
<i>PPH21 PPH22</i>	1.55 ± 0.05	1.85 ± 0.05	2.00 ± 0.00	116 ± 0.004	60.5 ± 3.4	r	+++
<i>pph21::LEU2 PPH22</i>	1.77 ± 0.18	1.97 ± 0.20	2.10 ± 0.27	124 ± 6.9	50.3 ± 1.6	r	++
<i>PPH21 pph22::URA3</i>	1.53 ± 0.03	1.77 ± 0.03	2.03 ± 0.15	121 ± 0.8	53.4 ± 0.4	r	++

The strains studied in Table III were also examined for the properties summarized in this table, mean values for the two strains of each *PPH* genotype being presented as appropriate. Sensitivity to  $\alpha$ -factor was scored as the growth inhibition zone size around discs containing differing levels of  $\alpha$ -factor. Doubling time and budding index (percentage budded cells) were measured using asynchronous, exponentially growing yeast cultures in YPD rich medium at 28°C.

r, resistant.

**Table V.** Yeast strains

Strain	Genotype
ASY100	<i>MAT<math>\alpha</math> ura3 leu2 HIS3 TRP1 ade pph21::LEU2</i> <i>MATa ura3 leu2 his3 trp1 ADE PPH21</i>
ASY101	<i>MAT<math>\alpha</math> ura3 leu2 HIS3 TRP1 ade pph21::LEU2 PPH22</i> <i>MATa ura3 leu2 his3 trp1 ADE PPH21 pph22::URA3</i>
LL20	<i>MAT<math>\alpha</math> leu2-3,-112 his3-11,-15</i>
MSY111	<i>MAT<math>\alpha</math> ura3 leu2 HIS3 TRP1 ade</i> <i>MATa ura3 leu2 his3 trp1 ADE</i>

*PPH22* can account for ~84% of the total PP2A activity in budding yeast. Although there are clearly no other PP2A genes which can fulfil the roles of *PPH21* and *PPH22* in their absence, the existence of other PP2A genes whose products might be expressed at different times or targeted to different substrates cannot be ruled out. Such activities might be readily detectable in our assay system *in vitro* but not capable of performing the functions of *PPH21* and *PPH22* *in vivo*. However, we do not consider that the residual 16% activity *does* reflect the presence of a third PP2A homologue in yeast for a variety of reasons. In the first instance, there may be other protein phosphatases in yeast which share with PP2A the property of exquisite sensitivity to okadaic acid but which are not strictly PP2A homologues. *SIT4* (Arndt *et al.*, 1989) might be a good candidate for such a protein phosphatase, showing some sequence similarity to PP2A but of unknown biochemical properties. Secondly, we cannot rule out that deletion of one of the two *PPH* loci is without effect on the activity of the second *PPH* locus. Such effects could be either transcriptional (e.g. if the cell was able to elevate the expression of either *PPH* locus in response to reduced PP2A level) or post-transcriptional (e.g. if total PP2A activity was normally limited by availability of regulatory subunits, but catalytic subunits were no longer in excess following inactivation of one *PPH* gene). Thirdly (and most importantly), our genomic Southern analyses clearly indicate that there are no other sequences which are closely related to *PPH21* and *PPH22*. We therefore consider it highly likely that there are just two PP2A genes in budding yeast.

### The *PPH* polypeptide amino-terminal extensions

Unlike the yeast PP1 genes (Ohkura *et al.*, 1989), both *PPH* genes appear to encode polypeptides much larger than their mammalian counterparts. Indeed, all other reported PP1 and PP2A catalytic subunits fall in the range 302 (*Drosophila*

PP1; Dombrádi *et al.*, 1989) to 337 (mouse PP1; Ohkura *et al.*, 1989) residues. The extra residues are located exclusively at the amino terminus of the predicted *PPH* gene products when compared with mammalian PP2A (Figure 3) and include both a region conserved between *PPH21* and *PPH22* and another (at the extreme amino terminus) which is not. However, amino-terminal extensions are a feature of several other yeast genes involved in regulatory pathways, for example the *BCY1* and *TPK* genes encoding the subunits of the cyclic AMP dependent protein kinase (Toda *et al.*, 1987a,b). Proteins which are destined for cellular locations other than the cytosol are often synthesized with amino-terminal extensions which constitute targeting signals to various organelles. However, neither *PPH* amino-terminal region appears to have the features expected for secreted (Kaiser *et al.*, 1987), mitochondrial (Roise *et al.*, 1988) or nuclear-localized proteins (Underwood and Fried, 1990), suggesting that the 'extra' regions encoded by *PPH21* and *PPH22* do not serve such a function. One possibility, therefore, is that these regions represent sequences required for the interaction of the PP2A catalytic subunits with yeast specific substrates or regulatory proteins. Mammalian PP2A is known to bind to such regulatory subunits, while in the case of PP1 various 'targeting' subunits appear to function by modulating its localization in the cell and may enhance its activity towards particular substrates (see Cohen, 1989 for a review). Similar regulatory subunits may be required for the *PPH* gene products to function correctly.

#### Possible roles for the *PPH* gene products

On the basis of other work we have suggested that the *PPH* gene products might play a role in cell cycle control. Since the double *pph21 pph22* null is inviable, we cannot by definition directly examine the properties of cells lacking PP2A activity. Such analysis must await the construction of conditional loss of function alleles of one or both genes so that both *PPH* genes can be inactivated in growing cells by, for example, shifting a *pph21 pph22<sup>ts</sup>* strain to the non-permissive temperature. Spores carrying the double *pph21 pph22* deletion germinated and grew for up to about five generations, suggesting that spores might inherit variable amounts of PP2A from the diploid parent and that the protein may be quite stable. Since the germinating spores did not generally show an arrest in the first one to two cell division cycles, it is difficult to attach significance to the asynchronous arrest phenotype observed and the data are not incompatible with PP2A having specific roles in cell cycle regulation. The effect of reduced PP2A activity on various cellular responses using the single *pph* strains failed to reveal any major change in the physiology of the yeast cells but indicated that *pph* strains have a slightly lengthened G<sub>1</sub> phase. While this result might appear to conflict with the role of PP2A as an inhibitor of mitotic induction in *Xenopus* egg extracts (Félix *et al.*, 1990), the absence of a G<sub>1</sub> phase in the latter system precludes a direct comparison. Furthermore, in strains lacking all PP2A activity a different phenotype may result.

It is now clear that regulation by protein phosphorylation is a critical element in yeast not only in the cell cycle but also for the response and adaptation to mating pheromones (see Elion *et al.*, 1990), the response of the cell to nutrient levels via the adenylate cyclase pathway (see Toda *et al.*, 1987a,b), control of transcriptional initiation (see Arndt *et al.*, 1989) and probably many other phenomena. Thus PP2A

activity potentially could be important in any of these processes. Given the extreme similarity of the predicted *PPH* gene products and the properties of the single *pph* strains, we conclude that the PPH21 and PPH22 proteins are largely interchangeable and that modest reduction in PP2A activity is insufficient to perturb greatly its essential cellular function(s).

## Materials and methods

#### Strains, media and general methods

All strains used and generated in the course of this work are summarized in Table V. Routine recombinant DNA methodology was performed according to Maniatis *et al.* (1982). Southern blots were produced using Hybond-N membranes (Amersham) and 0.8% agarose gels. High specific activity DNA hybridization probes were prepared by the random hexamer priming method (Feinberg and Vogelstein, 1983) using [ $\alpha$ -<sup>32</sup>P]dATP (>3000 Ci/mmol; Amersham). Basic yeast methods and media were as described by Sherman *et al.* (1986) unless stated otherwise. Transformation of yeast was as described by Ito *et al.* (1983). Yeast genomic DNA was prepared as described previously (Stark and Milner, 1989). Tetrad analysis was performed using a Leitz micromanipulator and using Zymolyase 100T (Seikagaku) to digest the asci. Chromosomal localization of the *PPH* genes by hybridization utilized a yeast genomic Chromoblot (Clontech).

#### Construction of a mini-library of *XbaI* restriction fragments for the isolation of *PPH* genomic clones

Genomic DNA from LL20 was digested to completion with *XbaI*, separated by agarose gel electrophoresis and DNA fragments in the size range 4.2–5.0 isolated by excising the relevant region of the gel. After recovery of the DNA by standard procedures, it was ligated into Bluescript KS M13– (Stratagene) which had previously been cut with *XbaI* and treated with calf intestinal alkaline phosphatase (BCL) to prevent recircularization. Transformation into *Escherichia coli* yielded ~800 independent clones.

#### DNA sequencing and analysis

All DNA sequencing was performed using the dideoxy method (Sanger *et al.*, 1977) using Sequenase 2.0 (United States Biochemical Corporation) and employing double-stranded DNA templates according to the Sequenase 2.0 protocol. Sequences were largely derived from overlapping subclones of the *PPH* genes using standard primers, employing synthetic oligonucleotide primers and the full length clones where required to complete overlaps. In each case both strands were fully sequenced. Subclones of the *PPH21* gene were derived from both the original AS3c cDNA and the subsequently isolated AS3g chromosomal sequence (see text): in Figure 2, bases 1–168 were derived from AS3g, bases 416–1223 were derived from AS3c and positions 169–415 and 1224–1286 were determined from both sources. In the region of the *PPH21* sequence determined exclusively from the cDNA the restriction map was precisely colinear in both the AS3c and AS3g inserts. DNA sequence assembly and analysis utilized the Staden and UWGCG molecular biology software (Staden, 1982; Devereux *et al.*, 1984).

#### *PPH* gene specific hybridization probes

A 233 bp *HpaII*–*EcoRI* fragment from the 5' end of the AS3 genomic clone was used as a probe for *PPH22*, while a 314 bp *KpnI*–*DraI* restriction fragment from the 5' end of the AS6 genomic clone was used as a hybridization probe for *PPH21*. Both these probes were totally specific for their respective *PPH* genes at the stringencies used. The probe used for the initial screening was a 0.96 kb *HpaI*–*RsaI* fragment extending from 20 bp before the initiator ATG to codon 303 of rabbit PP2A (da Cruz e Silva *et al.*, 1987).

#### Genetic disruption of the *PPH* loci

To generate the *pph21::LEU2* construct, the 2.9 kb *BglII* fragment from YE213 carrying *LEU2* was inserted into the *BglII* site of *PPH21*. This utilized an *EcoRI* fragment of the AS3c cDNA clone carrying most of the *PPH21* gene and which was inserted into Bluescript KS M13– (in AS3c, there is an *EcoRI* site nine bases beyond the sequence presented in Figure 2A). The *pph22::URA3* construct was made by isolating a 1.1 kb *HindIII* fragment carrying the *URA3* gene from YCp50 and inserting it into the *EcoRV* site of *PPH22* after it had been made blunt-ended by treatment with the Klenow fragment of T4 DNA polymerase and dNTPs (see Figure 6). This utilized an *AvaI*–*SacI* fragment from AS6g inserted into Bluescript KS M13–. The *pph21::LEU2* allele was excised as a *BamHI*–*XhoI* fragment using flanking polylinker sites in the construct and used to transform the



diploid MSY111 to leucine prototrophy. Genomic Southern blot analysis using *PPH21* and *LEU2* gene specific probes indicated the disruption of a single allele of *PPH21* in the transformed diploid strain (ASY100). In a second step, the *pph22::URA3* construct was similarly introduced into the genome of ASY100, excising the relevant region as a *Bsa*AI–*Dra*I fragment. *PPH22* and *URA3* gene specific probes were used to demonstrate that a single homologue of *PPH22* had been converted to *pph22::URA3*. Following tetrad analysis, selected haploid strains deduced to carry *pph* null alleles were again checked by Southern analysis using *PPH21* and *PPH22* gene specific probes to confirm the status of their *PPH* loci.

#### Protein phosphatase assays

These were performed on diluted yeast extracts essentially as described by Cohen *et al.* (1989b) using phosphorylated rabbit glycogen phosphorylase as a substrate, except that assays were incubated for 15 rather than 10 min. <sup>32</sup>P-Labeled substrate (240–380 c.p.m./pmol) was prepared by phosphorylation of phosphorylase with phosphorylase kinase using [ $\gamma$ -<sup>32</sup>P]ATP (1 mCi/mmol) as previously described (Cohen *et al.*, 1989a). PP2A was measured as activity independent of divalent cations which could be inhibited by 2 nM okadaic acid, while PP1 was measured as divalent cation independent activity expressed at 2 nM but not 2  $\mu$ M okadaic acid. For each extract, specific activities of PP1 and PP2A were determined by normalization of the activity using protein concentrations determined using the Bradford assay (BioRad).

#### Alpha-factor and heat-shock sensitivity

Sensitivity to  $\alpha$ -factor was determined essentially as described by Elion *et al.* (1990) by scoring the growth inhibition zone size (after 40 h at 28°C) around antibiotic assay discs (Whatman) containing 5–20  $\mu$ g of  $\alpha$ -factor (Sigma) placed on lawns of suitable *MATa* strains. Heat-shock sensitivity was examined by patching strains onto YPD plates (prewarmed to 55°C), incubating for 45 min at 55°C and then transferring the plates to 28°C for 2 days prior to scoring growth. In control experiments, this treatment abolished viability of strains carrying *bcy1* null alleles, in which hyperphosphorylation by cyclic 3',5'-AMP dependent protein kinase leads to heat-shock sensitivity.

#### Acknowledgements

Thanks are due to Alan Boyd for the *SEC1* gene, John Doonan for providing the *bimG* clone, Paul Dent for preparation of the protein phosphatase substrate, to Uta Präkelt and Peter Meacock for provision of the cDNA library, and to Philip Cohen. This work was supported by a grant from the Cancer Research Campaign (to M.J.R.S. and P.T.W.C.).

#### References

- Arndt, K.T., Styles, C.A. and Fink, G.R. (1987) *Science*, **237**, 874–880.
- Arndt, K.T., Styles, C.A. and Fink, G.R. (1989) *Cell*, **56**, 527–537.
- Axton, J.M., Dombrádi, V., Cohen, P.T.W. and Glover, D.M. (1990) *Cell*, **63**, 33–46.
- Bennetzen, J.L. and Hall, B.D. (1982) *J. Biol. Chem.*, **257**, 3026–3031.
- Berndt, N., Campbell, D.G., Caudwell, F.B., Cohen, P., da Cruz e Silva, E.F., da Cruz e Silva, O.B. and Cohen, P.T.W. (1987) *FEBS Lett.*, **223**, 340–346.
- Cohen, P. (1989) *Annu. Rev. Biochem.*, **58**, 453–508.
- Cohen, P.T.W. (1990) In Randle, P.J. (ed.), *Genetics and Human Nutrition*. Libbey and Co., London, in press.
- Cohen, P. and Cohen, P.T.W. (1989) *J. Biol. Chem.*, **264**, 21435–21438.
- Cohen, P., Klump, S. and Schelling, D.L. (1989a) *FEBS Lett.*, **250**, 596–600.
- Cohen, P., Schelling, D.L. and Stark, M.J.R. (1989b) *FEBS Lett.*, **250**, 601–606.
- Cohen, P.T.W., Brewis, N.D., Hughes, V. and Mann, D.J. (1990) *FEBS Lett.*, **268**, 355–359.
- Cyert, M.S. and Thorner, J. (1989) *Cell*, **57**, 891–893.
- da Cruz e Silva, O.B., Alemany, S., Campbell, D.G. and Cohen, P.T.W. (1987) *FEBS Lett.*, **221**, 415–422.
- da Cruz e Silva, O.B., da Cruz e Silva, E.F. and Cohen, P.T.W. (1988) *FEBS Lett.*, **242**, 106–110.
- Devereux, J., Haerberli, P. and Smithies, O. (1984) *Nucleic Acids Res.*, **12**, 387–395.
- Dombrádi, V., Axton, J.M., Glover, D.M. and Cohen, P.T.W. (1989) *Eur. J. Biochem.*, **183**, 603–610.
- Dombrádi, V., Axton, J.M., Barker, H.M. and Cohen, P.T.W. (1990) *FEBS Lett.*, in press.
- Doonan, J.H. and Morris, N.R. (1989) *Cell*, **57**, 987–996.

- Elion, E.A., Grisafi, P.L. and Fink, G.R. (1990) *Cell*, **60**, 649–664.
- Feinberg, A.P. and Vogelstein, B. (1983) *Anal. Biochem.*, **132**, 6–13.
- Félix, M.-A., Cohen, P. and Karsenti, E. (1990) *EMBO J.*, **9**, 675–683.
- Gallwitz, D., Halfter, H. and Mertins, P. (1987) In Kinghorn, J.R. (ed.), *Gene Structure in Eukaryotic Microbes*. IRL Press, Oxford, pp. 27–40.
- Hamilton, R., Watanabe, C.K. and de Boer, H.A. (1987) *Nucleic Acids Res.*, **15**, 3581–3593.
- Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) *J. Bacteriol.*, **153**, 163–168.
- Kaiser, C.A., Preuss, D., Grisafi, P. and Botstein, D. (1987) *Science*, **235**, 312–317.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Mortimer, R.K. and Schild, D. (1982) In Strathern, J.N. *et al.* (eds), *The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 11–26.
- Ohkura, H., Kinoshita, N., Miyatani, S., Toda, T. and Yanagida, M. (1989) *Cell*, **57**, 997–1007.
- Roise, D., Theiler, F., Horvath, S.J., Tomich, J.M., Richards, J.H., Allison, D.S. and Schatz, G. (1988) *EMBO J.*, **7**, 649–653.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
- Sherman, F., Fink, G.R. and Hicks, J.B. (1986) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Staden, R. (1982) *Nucleic Acids Res.*, **10**, 4731–4751.
- Stark, M.J.R. and Milner, J.S. (1989) *Yeast*, **5**, 35–50.
- Toda, T., Cameron, S., Sass, P., Zoller, M., McMullen, B., Hurwitz, M., Krebs, E.G. and Wigler, M. (1987a) *Mol. Cell. Biol.*, **7**, 1371–1377.
- Toda, T., Cameron, S., Sass, P., Zoller, M. and Wigler, M. (1987b) *Cell*, **50**, 277–287.
- Underwood, M.R. and Fried, H.M. (1990) *EMBO J.*, **9**, 91–99.

Received on July 31, 1990; revised on September 13, 1990

#### Note added in proof

The *PPH21* and *PPH22* DNA sequences reported here have been deposited with the EMBL sequence database under the respective accession numbers X56261 and X56262. We are grateful to Dr Hans Ronne for pointing out a minor error in the *PPH22* sequence prior to publication.